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NERVE REGENERATION IN THE COCKROACH, *LEUCOPHAEA MADERAE*: THE EFFECT OF HUMORAL SUBSTANCES *IN VITRO*

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The process of regenerating lost appendages (epimorphosis) was divided by Needham (1952) into three stages: wound closure, demolition of damaged cells and provision for defense, and differentiation of cells to provide new tissues for the repair process. This last stage was further divided into three parts: the formation growth, and differentiation of the regenerate.

The first two stages (wound closure and demolition and defense) follow leg extirpation in immature and adult insects. These processes as they occur *in vivo* were described in detail for epidermal tissues (Wigglesworth, 1957) and nerve tissue (Bodenstein, 1957; Guthrie, 1962). However, the formation and differentiation of the regenerate leg occurs only under limited circumstances. O'Farrell and Stock (1953) demonstrated that in Blattella nymphs, regenerate formation can occur only during the earlier portion of the molting cycle. When legs are removed during the last $\frac{1}{3}$ of the cycle, wound healing occurs and a papilla-like structure is formed, but no regenerate develops until the subsequent period of intermolt. Bodenstein (1955) also pointed out that adult cockroaches that normally cannot regenerate lost appendages do so when forced to molt by the implantation of active prothoracic glands and corpora cardiaca.

Penzlin (1965) showed that development of the regenerate was closely related to the molting cycle: Development is slow when the leg is removed early in the cycle and is rapid when the leg is removed late in the cycle. A critical point is finally reached, and no development occurs until the next molting cycle. Clearly then, the development of the leg regenerate is closely linked with the molting cycle, and the process of wound healing is not. Schneiderman and Gilbert (1964) pointed out that little is known about the mechanisms that control wound healing and regenerate formation and by what means the latter is linked to the molting cycle.

Penzlin (1963) described the development of the regenerate leg of *Periplaneta* as it is formed *in vivo* and in a second paper (1964), he outlined the role of the nervous system in the formation of the regenerate. When the leg is removed at the trochanter-femoral joint, the proximal stump of the fifth mesothoracic nerve that remains in the coxa becomes the source of new neurons that migrate into the developing regenerate. As the latter develops, it grows upward to enclose the distal portion of the old nerve stump.

The ability to maintain cockroach leg regenerates *in vitro* was reported by Marks and Reinecke (1964). The effect of various endocrine gland explants on

the development of cockroach leg regenerates was studied by the same authors (1965) who showed that the effects produced on epidermal tissues was quite different from those produced on nerve tissue. They suggested that the differences might be representative of the differences between the regenerate formation and wound healing processes and that such a system might be used to study these two processes. In a subsequent detailed study of the effects of various endocrine glands on epidermal tissues, Marks (1968) showed that these tissues did indeed respond quantitatively to prothoracic gland incubates, and this response could be modified by various *in vitro* endocrine gland interactions.

Marks, Reinecke and Leopold (1968) demonstrated that nerve regeneration *in vitro* was comparable to that reported from *in vivo* studies by Bodenstein (1957). These authors also confirmed that regenerative nerve growth does not appear to respond to the presence of the prothoracic gland in the same culture. Although these findings indicated that regenerating nerve tissue follows the wound healing pattern of growth and is controlled by a different mechanism from that which controls growth in epithelial tissues, a number of important questions remained unanswered, namely:

1. Are the effects on peripheral nerve growth produced by the presence of the ganglion in the culture unique to that organ or will nonendocrine tissues produce similar effects?
2. What effect is produced by other endocrine tissues and tissue combinations?
3. Must the endocrine tissues be present in the same chamber as the leg regenerate to produce an effect or is some kind of diffusible material produced that can give the same results as the gland itself?
4. Do interactions occur among the gland explants so that the effect produced by one gland is influenced by the presence of other glands?

Materials and Methods

A chemically defined (M-7) medium was used because it has the ability to support nerve growth in the absence of fetal calf serum (Marks and Reinecke, 1965). The preparation of the leg regenerates, ganglia, and glands was the same as that described in detail by Marks (1968). The mesothoracic legs of late instar nymphs were removed 24 hours after molting. Eight days later, the regenerates with the proximal stump of the fifth mesothoracic nerve protruding from them were dissected from the coxal stump, rinsed in nutrient medium, and placed under dialysis strips in Rose multipurpose chambers (3 or 4 to a chamber). They were arranged so that the nerve stumps were about 1 millimeter apart (Fig. 1). The endocrine glands and other tissues were removed at the same time and placed in tubes containing 1 cc of the same medium. The tubes and chambers were incubated for 6 days at 28° C. Then the chambers were checked for evidence of regenerative growth from the nerve stump with a phase contrast microscope. The emergence of glial cells from the stump of the fifth mesothoracic nerve accompanied by axons with characteristic growth cones was used as the criterion of regenerative growth since the appearance of such cells was normally followed by the linking of two explants (Marks *et al.*, 1968). If evidence of spontaneous growth was found, the leg regenerate was removed from consideration.

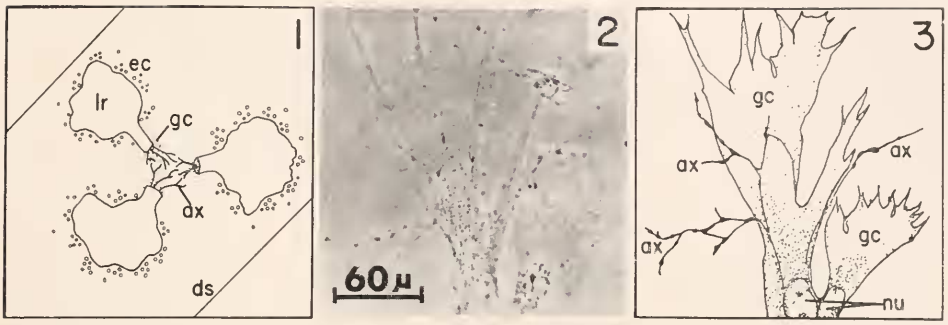

FIGURE 1. Diagram showing the arrangement of leg regenerate explants in the culture chamber. Epithelial cells (ec) surround the leg regenerate (lr) while glial cells (gc) and axon fibers (ax) emerge from the stump of the fifth mesothoracic nerve. The entire preparation is held in place by a strip of dialysis membrane (ds).

FIGURE 2. Glial cells with accompanying axon fibers emerging from the nerve stump of a cockroach leg regenerate. Dark contrast phase; 25 days *in vitro*.

FIGURE 3. Diagrammatic representation of the photograph in Figure 2. Axon fibers (ax) lying on the surface of glial cells (gc) extend out onto the surface of the cover glass. The granules that surround the nucelus (nu) probably contain glycogen.

The chambers were emptied and refilled with the medium in which the glands had been incubated, and the gland itself was discarded. After 6 more days of incubation, the chambers were again checked, and if glial cells and axons were present, the explant was scored as positive; if they were not present, it was scored as negative (Fig. 2).

Three sets of controls were used. In one set, fresh medium was added after the initial reading; the second set contained fresh medium to which was added 5% fetal calf serum; and in the third set, 1 cc of medium was added in which a 2-mm cube of cockroach muscle tissue had been incubated. The results obtained with gland incubates were tested against the controls by using the test for the difference between two sample proportions given by Goldstein (1964); significance was set at $P = 0.05$.

RESULTS

Control series

If the results of these experiments were to be meaningful, we first had to be sure that the changes in occurrence of regenerative nerve growth in the experimental chambers were not affected perceptibly by either nutrient substances or nonspecific growth factors released into the medium by the glands being tested. Therefore we ran a series of controls that incorporated three test media. The results are given in Table I.

When the chambers were refed with fresh nutrient medium, 9% of 47 regenerates showed evidence of regenerative growth from the nerve stump. When similar chambers were refed with fresh medium containing 5% fetal calf serum (a common tissue culture growth supplement), only 5% of 27 regenerates showed nerve growth. Also, when the third set of chambers was refed with nutrient

TABLE I

Control series showing the effect on nonspecific and nutrient culture medium additives on nerve regeneration in cockroach leg regenerates

Test medium	Per cent of explants showing nerve regeneration
M-7	9% of 47
M-7 + 5% fetal calf serum	5% of 27
M-7 muscle incubate (4 cu. mm muscle incubated for 6 days)	5% of 20

in which muscle tissue was incubated, only 5% of 20 showed nerve growth. These experiments indicate that the occurrence of nerve cell migration was not visibly influenced by the nutrient substances present in fetal calf serum and suggest that nerve growth is not influenced by nutritive factors supplied by the test incubates.

When the prothoracic ganglion was cultured instead of the leg regenerate, it responded to the presence of fetal calf serum with a six-fold increase in the occurrence of regenerative growth. This difference in the response of the two tissues to the presence of fetal calf serum was probably a consequence of the structure of the two organs. The leg regenerate at this stage of development is largely an epithelial sac filled with blood cells, and thus it carries a good supply of nutrient material into the culture chamber. On the other hand, the ganglion is essentially a solid organ and carries little if any blood into the culture. Thus, it is more sensitive to the presence of nutrient materials in the medium and is therefore a poor test organ for studies of this type.

It was also apparent from the control series that incubates of muscle tissue explants approximately the same size as the largest endocrine gland explants do not provide any nonspecific tissue factors that visibly effect nerve growth. Since the experimental design is such that the test tissues and the leg regenerates are never in the same culture at the same time, it can be assumed that any any influence produced by the test incubates on the leg regenerate must be caused by specific diffusible substances released by the gland explant into the culture medium during the period of incubation. It is thus possible to test the effects of incubates of various endocrine glands and gland combinations on nerve growth and to evaluate the results with some degree of confidence.

TABLE II

The effect of endocrine gland incubates on regenerative growth from the nerve stump of a cockroach leg regenerate

Tissue	Dose	Per cent of explants showing nerve regeneration
Prothoracic gland	1 gland/cc	0% of 23
	2 glands/cc	3% of 33
	3 glands/cc	0% of 21
Corpus allatum cardiacum complex	2 glands/cc	0% of 26
Brain	2 glands/cc	14% of 22
Ganglion	2 ganglion/cc	25% of 24

TABLE III

The effect of mode of incubation on the effect of incubates on nerve regeneration in the cockroach leg regenerate

Tissue	Mode of incubation	Per cent of explants showing nerve regeneration
Gland + ganglion	Separately	35% of 23
Gland + brain	Separately	14% of 22
Gland + corpora allata-cardiacum	Separately	7% of 27
Gland + muscle	Together	4% of 26
Gland + ganglion	Together	16% of 18
Gland + brain	Together	0% of 26
Gland + corpora allata-cardiacum	Together	0% of 19

Effect of gland incubates

In the first tests, incubates from individual glands were tested for their effect on the growth of nerve tissue in the regenerating leg stump. The results are given in Table II. When the incubate of prothoracic glands was tested on leg regenerates, no measurable effect on nerve cell migration was produced, regardless of the number of glands used.

Incubates of the corpus allatum-cardiacum complex also showed no measurable effect. Incubates from the brain (though they produced a frequency of nerve growth that was significantly different from that produced by the prothoracic gland and the corpus allatum-cardiacum complex) showed no significant difference from the control series. Thus, while brain incubates were not inhibitory in effect, no definite stimulation could be demonstrated. In contrast, the incubate from prothoracic ganglia had a significant stimulatory effect on the frequency of regenerative growth.

In the second series of tests, combinations of the prothoracic gland with other glands were tested. Two kinds of combinations were used: those in which the glands were combined and incubated together and those in which the glands were incubated separately and the incubates then combined. The results are given in Table III.

When the various glands were incubated separately and the incubates combined, the results were effectively the same as those obtained with the same glands without the addition of prothoracic gland incubate. However, when these same glands were incubated together with the prothoracic gland, the stimulatory effect of the prothoracic ganglion was reduced by 54%, and the borderline effect of the brain incubate disappeared entirely. The only tissues that were not affected by the presence of the prothoracic gland during the incubation process were the corpus allatum-cardiacum complex and the muscle. Since the two series differed only in the mode of incubation of the glands, the test in which the glands were incubated separately served as a control for the second set of tests; as an additional control, a prothoracic gland was incubated with a piece of muscle tissue. When the incubate was tested, the results were the same as when these same tissues were tested separately (no evidence of interaction was evident during the incubation period).

Discussion

The migration of axons and glial cells from the nerve stump of the leg regenerate was related to nerve regeneration *in vitro* by Marks *et al.* (1968). Marks and Reinecke (1965) demonstrated that the presence of the prothoracic ganglion in the same chamber stimulated this cell migration. Our present experiments demonstrate conclusively that this stimulatory effect can be produced by a diffusible substance that is carried from one culture to another in the nutrient medium. The possible presence of a nerve growth stimulator in brain incubates as well suggests that the substance may be common to all ganglionic tissues in the central nervous system. Failure of either the prothoracic gland or the corpus allatum-cardiacum complex to produce an effect further suggests that the stimulation is probably nonendocrine. The nature of this substance is unknown. However, a nerve growth stimulator isolated from vertebrate sources was identified as a protein by Levi-Montalcini (1964).

In an earlier study of leg regenerate growth, Marks (1968) demonstrated that high titers of prothoracic gland secretion produced an inhibitory effect on regenerative processes in epidermal tissues. Similarly, O'Farrell and Stock (1953) and Penzlin (1965) demonstrated *in vivo* that regenerate formation did not occur during the latter part of the intermolt when the effect of molting hormone is presumably maximal. It is of particular interest, then, to know what effect the prothoracic gland has on the regenerative growth of nerve tissue.

When the brain, prothoracic ganglion, and allatum-cardiacum complex were incubated separately and the incubates combined with incubates of a single prothoracic gland, the failure of the various incubate combinations to produce an effect different from that produced by these same glands alone suggests that the prothoracic gland itself produced little or no effect on nerve growth *per se*. In addition to this series that served as a control for the next series of experiments, the gland-muscle combination gave similar results that eliminated the likelihood that nonspecific tissue factors might affect the interaction among the glands.

When the ganglion and prothoracic glands were incubated together and the resulting incubate was tested, we found a sharp reduction in the frequency of nerve growth. When compared with the preceding experiment, the reduction clearly indicated that an interaction had occurred between the glands during the period of incubation. The result was a decrease in the frequency of regenerative nerve growth.

There are two possible explanations for these results: The first derives from the fact that the ganglion was demonstrated to stimulate the prothoracic gland *in vitro* (Marks, 1968). Thus, the increased titer of secretion from the prothoracic gland might inhibit nerve growth *per se* by acting directly on the nerve tissue. However, when the same glands were incubated separately, the frequency of regenerative activity produced was not decreased by the presence of prothoracic gland incubate. This hypothesis must thus be discarded.

The second explanation proposes that the prothoracic gland acts on the ganglion to inhibit its production of nerve growth factor and that it produces little or no direct effect on the nerve itself. This hypothesis not only accounts for the apparent lack of activity of the prothoracic gland secretion on the regenerate but it also explains why the brain and ganglion produced similar effects when allowed to

interact with the prothoracic gland. This explanation appears to be the correct one. The production of nerve growth factor by the ganglion is thus regulated, at least in part, by a diffusible substance secreted by the prothoracic gland.

Three conclusions can be drawn from these findings: (1) A diffusible substance is released by the prothoracic ganglion when it is incubated in a chemically defined nutrient medium. This substance increases the frequency of regenerative nerve growth in cockroach leg regenerates *in vitro*. (2) The prothoracic glands, the allatum-cardiacum complex, and the muscle tissue showed no such activity. (3) When the ganglion and prothoracic gland were allowed to interact, the stimulatory effect was reduced by more than 50%. The prothoracic gland apparently depressed the production by the ganglion of the growth-stimulating material. Assuming that these *in vitro* findings are representative of the process of nerve regeneration as it occurs *in vivo*, they can be related to the overall process of leg regeneration. Marks (1968) showed that a high titer of prothoracic gland secretion similar to that present during the last part of the molting cycle inhibits the development of a leg regenerate by acting on the epidermal tissues. Penzlin (1964) demonstrated that the development of the musculature of a leg regenerate is partially dependent on the previous regeneration of the nerve. Thus, the high level of prothoracic gland secretion that inhibits the development of epidermal tissues also retards the regeneration of nerve and indirectly of muscle tissue by inhibiting the production of nerve growth factor by the ganglion. It is scarcely surprising that under these conditions, a leg regenerate fails to develop until the following instar.

Summary

An *in vitro* system was used to study the effects of diffusible substances from various endocrine tissues on the growth of nerve tissue from regenerating cockroach legs. Incubates prepared from endocrine glands and gland combinations were tested for their effect on regenerative growth from the stump of the fifth mesothoracic nerve of 8-day leg regenerates from late instar nymphs of *Leucophaea maderae*. Incubates prepared from the prothoracic ganglion produced a stimulatory effect, while the prothoracic gland, allatum-cardiacum complex, and brain showed no effect. When prothoracic gland and prothoracic ganglion incubates were combined, the results were the same as with ganglion incubate alone. When the glands were incubated together and allowed to interact, the nerve growth stimulating effect of the ganglion was reduced by 50%. To explain these findings, it is postulated that a diffusible substance is released by the prothoracic ganglion which stimulates regenerative nerve growth from the leg regenerate. The release of this nerve growth stimulating factor is inhibited by interaction with the prothoracic gland. The possible effect of this interaction on the process of leg regeneration as it occurs *in vivo* is discussed.

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